Preparation of Serum Capped Silver Nanoparticles for Selective Killing of Microbial Cells Sparing Host Cells

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Preparation of serum capped silver nanoparticles for selective killing of microbial cells sparing host cells

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Abstract:

Following access into cell, colloidal silver nanoparticles exhibit generalized cytotoxic properties, thus appear as omnipotent microbicidal, but not suitable for systemic use unless are free of toxic effects on host cells. The serum capped silver nanoparticles when prepared from silver nitrate, using dextrose as reducing and group-matched homologous serum as a stabilizing agent, selective endocytosis and oxidative stress dependent bio-functional damages to the host are mostly eliminated. For their bio-mimicking outer coat, there is least possibility of internalization into host-cells or liberation of excess oxidants in circulation following interaction with erythrocytes or vascular endothelial cells. Presence of infection specific antibody in the serum can make such nano-conjugates more selective. A potent antimicrobial action and a wide margin of safety for mammalian cells in comparison with very similar PVA-capped silver nanoparticles have been demonstrated by in-vitro challenge of such nanoparticles on different microbes, human liver cell-line, and *in-vivo* study on mice model. This may open-up wide-range therapeutic prospects of colloidal nanoparticles.

Introduction:

Due to the rising trend of infections with “superbugs” where no antibiotics have left an option for effective management ¹, suitable nonconventional antimicrobial agents need to be developed. From the beginning of the penicillin era, bacteria have started developing resistance against all newly introduced antibiotics within a few decades. Bacteria accomplish this by a few simple genetic or structural alterations under selective pressure at the target level where the drug could arrest cell replication. So, instead of developing few more selective anti-replicant drug to combat superbugs, a group of resistance-proof, multi-targeted, potent, wide-range cytotoxic agents can be a permanent solution of the issue, provided they spare entry into host cells during systemic use.

By in-vitro studies, almost all heavy metal-based antimicrobial nanoparticles (NP) have shown dose-dependent pan-microbicidal actions against all tested bacteria² fungi³, viruses⁴ or parasites⁵ depending on extent of intracellular access. Their unique mechanisms of internalization into microbial and mammalian cells lead to disruption of cellular functions by reacting on different biomolecules at unusual reduced atomic materialistic state ^{6,7}. These have made them near-ideal new-age antimicrobial agents. However, in the question of the margin of safety, none are suitable for systemic use or environment friendly. For the same reason, their uses remain restricted to some topical applicable drugs for wound management ⁸, biofilm-proof indwelling device coating or use as food preservatives, antiseptics and pesticide agents.

An attempt has been made to prepare a systemic usable silver nanoparticle (AgNPs) containing a robust core of aggregated reduced silver atoms (Ag^0), stabilized by homologous serum components. In circulation, the host's serum can impart further stability and may not allow release of toxic core materials before internalization into target cells. This is like “targeted nano-missile with a pay-load cluster of atoms”. Due to high zeta-potential, these will be more attracted towards rapidly multiplying, negatively charged bacteria, regenerating cells or cancer cells and will be internalized by forced splitting of the bacterial bi-layered lipid membrane, while may fail to be endocytosed into normal host cells due to receptor-ligand recognition inadequacy by self-proteins capped particles.

In blood circulation, other nanoparticles are likely to confront first with negatively charged plenty of oxidant-full erythrocytes and vascular endothelial cells. This may cause endocytosis after non-specific or blood-group antigen specific attachment to erythrocytes, resulting dose-dependent hemolysis or micro-agglutination followed by erythro-phagocytosis. Thus, excess ROS liberated into circulation may cause cytotoxicity on different host tissues after dissemination through circulation. By using blood group compatible serum as capping agent such risk can be averted. Their in-vitro and in-vivo antimicrobial activities along with the margin of safety for therapeutic applications need to be established before going for trial on infected animal model or human volunteers.

Results

Characterization of test and control AgNPs:

The maximum absorption peak of serum stabilized silver nano-antimicrobial displayed by UV-Vis absorption spectra was around 410 nm and peak width 20nm [Fig.1] Their cluster size distribution obtained from DLS along with negative zeta potential values -8.9 mV ensured high aggregation stability of such mixture of AgNPs in aqueous dispersion. AgNPs were almost round in shape and average size was 18 nm as demonstrated by transmission electron microscopy (TEM) images.

Fig.1. Physical characterization of serum capped silver nanoparticles (A) UV–Vis absorption spectra of AgNPs (B) Cluster size obtained from DLS measurements (C) Zeta potential of AgNPs. (D) TEM image

The maximum absorption peak of control PVA-capped silver nano-antimicrobial displayed by UV-Vis absorption spectra was around 420 nm [Figure 2.] Their cluster size distribution obtained from DLS along with negative zeta potential values -8.8 mV ensured high aggregation stability of such mixture of AgNPs in aqueous dispersion. AgNPs were almost round in shape and average size was 13 nm (2.D) as demonstrated by transmission electron microscopy (TEM) images.

Fig.2. Physical characterization of PVA capped silver nanoparticles (A) UV–Vis absorption spectra of AgNPs (B) Cluster size obtained from DLS measurements (C) Zeta potential of AgNPs (D) TEM image

Antimicrobial assay

The comparative MIC values showed 1280 (0.836 $\mu\text{g/mL}$), 1280, 1280 and 640 (1.67 $\mu\text{g/mL}$) folds higher antimicrobial action respectively for test AgNPs [Fig. 3: Bar-diagram of respective log₂ dilution factors at MICs] than those of equivalent ionic silver solutions for tested *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans*. The term “bonus effect” was applied here as the “biological marker” of the nano-antimicrobial, mainly due to added antimicrobial action of Ag^0 by creating oxidative stress, which was > 500 for all test organisms. Thus, for all practical purpose inhibition of any one of these bacteria (as reference strain) at concentration $< 2 \mu\text{g/mL}$ silver can be taken as nano conversion of silver without confirmation by complicated physical parameters. MIC values both for sensitive reference strains and MDR strains of any bacterium were almost equal within range of biological errors for particular NPs, which indicated genetically acquired drug resistance for evading a target intervention of microbial replication didn’t come on the way of nano-antimicrobial cytotoxic action.

Fig.3: Added anti-microbial action of silver by shifting MIC (log₂ MIC dilution factors or at 2, 4, 8,...256, 512 dilutions) following nano conversion [Blue bar ~ 1/10th diluted equivalent Ag⁺ solution]

Preservation of AgNPs:

MIC values against test reference strain bacteria were noted 2 folds higher after 6 months at 4°C refrigeration storage, whereas remained unchanged even at 6 months when preserved at room temperature in an oxygen-depleted environment. Inert gas filled sealed amber coloured container can be used for the purpose without the need of oxygen-depleted container for preservation. This indicated a fair degree of stability of such serum-capped cocktail nanoparticles, at least for 3 months.

MTT assay on normal human liver cell line (WRL 68)

MTT assay results showed almost no toxic effects of serum-capped AgNPs on human liver cell viability even at much higher concentration (500 µg/mL) required for antimicrobial therapy, whereas higher toxicity was noted after using AgNP-PVA-13 even at 1/10th lower concentration.

Fig.4. MTT Assay. (A) Normal liver cell line (WRL-68) with AgNP-Serum-18; (B) Normal liver cell line (WRL-68) with AgNP-PVA-13.

Colony forming unit (CFU) assay on WRL 68 Cell line

The extent of inhibition in terms of CFU reduction after treatment with different concentrations of AgNPs compared with control demonstrated almost no inhibition at a concentration of AgNPs as high as 200 mg/L silver equivalent, and 50th percentile inhibition at < 500 µg/mL silver equivalent concentrations [Fig. 5]. Though by MTT assay at same highest concentration showed almost no cell death, might suppress cell replication to many cells on prolong exposure.

Same inhibition study was performed for PVA capped silver nanoparticles, where 50th percentile inhibition at >10 mg/L silver equivalent concentrations indicating at least 50 folds higher toxicity.

Fig.5. CFU assay. A. Image of a normal human liver cell line (control); B. Cell line with the treatment of AgNPs- Serum-18 at 100 µg/mL silver equivalent concentration; C. Cell line with the treatment of AgNPs at 200 µg/mL silver equivalent concentration; D. Cell line with the treatment of AgNPs at 500 µg/mL silver equivalent concentration; E. Inhibition of colony formation on normal human liver cell line after treatment of AgNPs at various concentrations.

Cytotoxicity study in mice model:

No behavioral change of tested mice nor histopathological changes in liver tissue, kidney and brain tissue [Fig. 6] were observed in a preliminary study for cytotoxicity on mice model even in 3570 µg silver equivalent test serum capped AgNPs /kg body weight I.V or 7140 µg/kg body weight I.P injected mice. A similar observation was reported using bovine serum capped nanoparticles in the rat model⁹. It also reiterates that human serum capped AgNPs at low dose is well-tolerated in the murine model. For technical reason, the test could not be carried out with mouse serum capped AgNPs and at higher dose of nano preparations to obtain end point toxicity evaluation. The cytotoxic effects of non-serum capped nanoparticles (PVA capped) were pronounced and all tested mice at 357 µg/kg test dose (1/10th.) died within 1st week with focal hemorrhage and necrosis in different organs [Fig.6]. Results indicated appreciable safety limit

(> 10) of serum-capped AgNPs. similar toxic effects were noted with most other non-serum capped nanoparticles¹⁰, though studies showed gradual diminution of their bio-functional properties in blood circulation by acquiring corona protein coat.

Fig.6. Hematoxylin and eosin (H&E)-stained liver and brain section from mice in different groups. (A) Liver section of mice in the control group showing normal histology and hepatocytes arranged in the hepatic cords. (B) Liver sections of mice in the group that received 3570 $\mu\text{g}/\text{kg}$ AgNP-Serum-18 (I.V), showing: normal histology (C) Liver of mice in the group that received 357 $\mu\text{g}/\text{kg}$ AgNP-PVA-13 showing hepatocellular vacuolation and focal area of hemorrhage in the hepatic parenchyma. (D) Brain section of mice in the control group showing normal histology. (E) Brain sections of mice in the group that received 3570 $\mu\text{g}/\text{kg}$ AgNP-Serum-18 (I.V), showing: normal histology (F) Brain of mice in the group that received 357 $\mu\text{g}/\text{kg}$ AgNP-PVA-13 showing signs of early neuronal injury, focal inflammatory cellular infiltration and vascular congestion. (G) Kidney section of mice in the control group showing normal glomerulus. (H) Kidney section of mice in the Serum capped AgNPs group showing no necrosis (I) Kidney section of mice in the AgNP-PVA-13 group showing necrosis found in kidney tubules.

Effect on Blood Biochemistry

100 μl of PVA capped and PVA capped nanoparticles were injected into the blood stream, blood biochemistry was evaluated. PVA capped nanoparticles showed significantly higher toxic effects on functions of different organs even at $1/10^{\text{th}}$ concentration, when compared to the control group and serum capped silver nanoparticles (Table 1).

Dose ($\mu\text{g/kg}$)	Control	Silver 3570 (Serum Capped)	Silver 357 (PVA Capped)
BUN ¹ (mg/dL)	109.75 $\pm$ 7.5	139 $\pm$ 12	280 $\pm$ 17
CRE ² (mg/dL)	0.45 $\pm$ 0.15	0.5 $\pm$ 0.1	1.675 $\pm$ 0.2
TBIL ³ (mg/dL)	0.225 $\pm$ 0.15	0.2 $\pm$ 0.1	1.025 $\pm$ 0.35
CBIL ⁴ (mg/dL)	0.02 $\pm$ 0.015	0.0325 $\pm$ 0.1	0.37 $\pm$ 0.36
UNCBIL ⁵ (mg/dL)	0.015 $\pm$ 0.005	0.035 $\pm$ 0.005	0.08 $\pm$ 0.01
ALP ⁶ (IU/L)	144.75 $\pm$ 14	148.5 $\pm$ 13.5	255.5 $\pm$ 26
AST ⁷ (IU/L)	164.75 $\pm$ 44	144.25 $\pm$ 16.5	378 $\pm$ 12.5
ALT ⁸ (IU/L)	63 $\pm$ 3.5	62.75 $\pm$ 6.5	73.5 $\pm$ 12

Table 1

1, blood urea nitrogen; 2, creatinine; 3, total bilirubin; 4, conjugated bilirubin; 5, unconjugated bilirubin; 6, alkaline phosphatase; 7, aspartate amino transaminase; 8, alanine amino transaminase.

Serum biochemical values of swiss albino male mice after 4 weeks of AgNP administration (n=4).

Discussion:

By in-vitro studies many heavy metal nanoparticles have been proved as wide range, potent antimicrobial agents¹¹, of which AgNPs have been extensively studied and believed as more stable, less toxic, broad-spectrum, apparently resistant-proof potent antimicrobial agents. During wet chemical preparation, the reducing agent gives electrons to silver ions (Ag^+) to form unstable silver atoms (Ag^0), which tend to form unstable clusters of uncapped silver nanoparticles. By electrostatic stabilization, surfactant polymers prevent the uncontrolled amount of particle formation and particle aggregation by steric repulsion, giving rise to nano-sized (1-100 nm) colloidal particles containing a core of aggregated silver atoms capped by stabilizing molecule with adsorption of ions to the surface. This creates an electrical double layer that impairs Columbic repulsion force between individual particles. That electrostatic force is expressed as Zeta potential of colloidal nanoparticles and is an important guiding force to interact with other cells with great differences of electrostatic surface charges.

Such nanoparticles can bind to the bacterial surface mainly by electrostatic forces¹² and split bi-layered lipid membrane with a special affinity to negatively charged lipopolysaccharide of Gram-negative bacteria^{13,14}. This, in turn, causes greater permeability, osmotic disbalance and release of cytoplasmic contents. Silver atoms attack the respiratory chains at mesosome level of the bacterial cell membrane, resulting in cell death. Such antibacterial activities are mainly mediated by reactive oxygen species (ROS) and found to be increased in the presence of oxygen and decreased in the

presence of antioxidants¹⁵. Similar oxidative stress by excess production of ROS after interaction with Ag⁰ can happen inside the mammalian cell at mitochondria level, through the reaction of silver with the thiol group of the enzymes¹⁶. Also, ROS can cause dephosphorylation of peptides on tyrosine residues and promotes cell death by influencing bacterial signal transduction^{7,17}. However, internalization of nanoparticles in that case predominantly occurs by endocytosis through receptor-ligand interactions. So, toxicity of nanoparticles to mammalian cells is primarily regulated by structure of surface capping molecules^{18,19}. Due to large surface area to volume ratios, large Ag⁰ payloads can be delivered by small size triangular silver nanoparticles than larger one²⁰. Their size and shape depend upon the method of nano preparation and used stabilizing molecules for the purpose.

The minor protein compositions of human serum differ from one individual to others in terms of blood group iso-antibodies, acquired immune-bodies following vaccination or infection and auto-antibody in persons with auto-immune disorders. Their inclusion as capping agents in serum capped AgNPs may have adverse effects by targeting selective host cells. This may explain dose-dependent hemolysis by polyvinyl pyrrolidone and citrate capped AgNPs²¹ with the observation of ROS mediated membrane lipid damage²², leading to hemolysis²³. If such nanoparticles target non nucleated human RBCs²⁴ devoid of most cytoplasmic organelle and release sufficient free Ag⁰ or ROS into circulation before attachment with target microbes, may also cause many unpredictable adverse effects to other cells of the host. For this reason, we recommend the use of matched serum preferably with antibody of specific infection. Disease-specific monoclonal antibodies can be added to make these more target specific microbicidal agents. Similarly, vascular endothelial cells with strong negative surface charge can be first target of positive-charged nanoparticles. As vascular wall is covered with endothelial glycocalyx supplemented with components of plasma, may restrict entry of plasma / serum capped nanoparticles. Thus, it may open-up the immense possibilities of therapeutic applications by a new class of nano-composites. Earlier workers also emphasized the role of surface capping agents of noble metal nanoparticles for molecular recognition of target cells²⁵. A nano-silver probe for immunoassay has also been developed by Yeh et-al²⁶ by using IgG antibody in citrated capped AgNPs. So, disease-specific antibodies on the surface of systemic usable AgNPs should be recommended for selective destruction of target cells or microbes.

Other factors for prohibiting systemic use of many non-serum-capped nanoparticles were, instability or short half-life, unmatched viscous colloidal state of the preparation and adsorption of various essential protein components from circulation^{27,28}. In the experiment with bovine albumin capped AgNPs, corona formation with protein molecules at their surface^{29,30} was observed and such AgNPs caused least hemolysis than others^{31,32}. Huang H et-al²¹ also demonstrated 20 different corona proteins after incubating citrate and PVP capped AgNPs with plasma, including immune-globulin fragments. So, self-serum or plasma was chosen as most suitable capping agent for systemic use not only to guard hemolysis but also for the selective attack to microbes against which person carries antibodies. Nanoparticles prepared from blood group matched serum of convalescence patients may also be used as life-saving infection controlling agent. During intravenous use of nanoparticles, nucleus and mitochondria free human RBCs are first target, being a bag of oxygen carrying molecules. So, AgNP induced ROS coming into circulation even after depletion of 0.1% cells, can cause sufficient toxic effects to the host by endothelial damage, platelet

aggregation, phagocytic oxidative burst and genotoxicity. This may be the major reason of toxicity for earlier nano preparations, though these become gradually less hemolytic after acquiring plasma derived corona coats. For same reasons, the test AgNP-Serum-18 is proved much safer than equivalent silver containing AgNP-PVA-13, by our various test parameters on mice and human liver cell line.

After human trials, therapy with appropriate safe serum coat nanoparticles can be introduced as life-saving measures in MDR bacterial or fungal infections (including MDR *Candida auris*, Mucor-mycosis) and some viral infections, like hepatitis B, AIDS, Ebola, COVID-19 etc. Even rabies patients may be treated without reversion of neural damages, by suitable nanoparticles capped with a mixture of self-serum and hyper-immune serum.

For instant preparation of such self-serum-based nano-antimicrobials, an automated vending machine may be designed where ingredient solutions can be mixed sequentially at required physical conditions.

Plasma or whole blood collected with dextrose-citrate solution may be similarly tested for nano-conversion. However, serum is preferred because nano-size and bio-functional efficacy may be compromised with further incorporation of coagulation factors on capping materials. Other potent heavy metal nano-antimicrobial particles may also be made therapeutically useful after treating with self-plasma/ serum in starring condition to convert into “secondary novel nanoparticles”. However, they may be toxic at uncapped state.

In earlier studies, different AgNPs showed nonspecific synergism³³⁻³⁵ with conventional antibiotics and potent anti-biofilm properties³⁶. This can be advantageously used in therapeutic planning of combination therapy for low dose use of both drugs. Alteration of cell membrane permeability in damaged nano treated microbial cells may be the primary reason for non-specific synergism for other drugs. As the mechanism of antimicrobial action of nanoparticles is independent of cell replication, these can also kill dormant, non-replicating microbes within biofilms. This may be an important reason for antibiofilm role and action on non-replicating cells like human RBC.

A proposed single-dose infusion of 100 mL serum/plasma capped AgNPs may be theoretically enough³⁷ for managing intractable infections in terms of higher MIC achievable concentration in blood for most in-vitro tested MDR strains, keeping the wide margin of safety for the host. Interpolating results of in-vitro and in-vivo toxicity study, the same is indicated. The amount of silver delivered with 100 mL AgNPs is only 10.8 mg (approximate achievable plasma concentration > 2 mg/L sufficient for antimicrobial action, while 0.1 mg silver / kg body weight well tolerable by the host). This, after oxidation within cells, are likely to be eliminated from body through kidney as ionic silver and may not be harmful for environment. Even by using 1/10th of AgNPs (10 mL), synergistically with one apparently resistant antibiotic, desired infection control may be possible. This is supported by an in-vitro study adding 1/4th MIC carboxy-methyl cellulose capped AgNPs at ultra-dilution in emulsion fluid of automated susceptibility testing device that reverted all resistant drugs into sensitive range³³. For preparation of this 10 mL AgNPs, only 2.5 mL patient's serum will be required, which can be easily collected from any adult patient.

Many workers claimed as safer nanoparticles when prepared by “green bio-synthesis” using various plant or microbial extracts as stabilizing agents. But such organic coating agents were only

suitable for topical use, not for systemic application³⁸. The test serum-capped AgNPs can also be considered as one type of green bio-synthetic products, but safer for systemic use.

Methods:

Chemicals and Biological Materials

For the synthesis of two different AgNPs, silver nitrate (99.9%, Sigma Aldrich, USA) was taken along with dextrose (Sigma Aldrich, USA) and sodium borohydride (Sigma Aldrich, USA) as reducing agent. As capping agent, pooled, “O” Rh-D positive blood group human serums from discarded samples of healthy individuals were taken as capping agents, after sterilization by passing through the membrane filter. Poly vinyl alcohol (PVA) (Hi-media, India) was used as a primary capping or anionic surfactant stabilizing agent for preparation of another AgNPs.

For the determination of respective MICs of serum capped AgNPs against different microorganisms, their Mueller-Hinton broth cultures (Hi-media, India) were used. The following standard reference strains microbes (Microbiologics-Inc, USA) were used: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 *Candida albicans* ATCC 10231. Clinical isolates of similar multidrug resistant microbes from blood samples of sepsis patients were included after isolation, identification and antimicrobial susceptibility testing in the automated system in our laboratory.

Preparation of systemic usable AgNPs

Wet chemical reduction method³⁹ was followed to prepare systemic usable colloidal AgNPs. Pooled, “O” Rh-D positive blood group human serums was used as a primary capping or anionic surfactant stabilizing agent and dextrose as a reducing agent. Actually, serum was used as a mixture of various capping protein molecules and some reducing substances. So, the presence of such cock-tail stabilizing molecules in excess only ensured excess stabilization of the product without increasing toxicity.

The aqueous solution of silver nitrate (AgNO_3) was prepared in deionized water at 4 mM concentration (672 mg/ L AgNO_3) so that effective concentration of silver in nano preparation resulted to 1 mM (106.8 mg/ L silver or 168 mg AgNO_3 /L) concentration. Five mL of serum (pH 7.4) was taken in 50 mL beaker. This serum stirred and heated at 56°C to obtain decomplexed serum solution. Then 5mL of 4mM AgNO_3 solution was added to the serum solution under continuous stirring to obtain Ag^+ /serum complex solution. 10 mL of 2mM dextrose solution was added to the Ag^+ /serum complex solution under continuous stirring. The temperature of solution increased to 75 °C. The optimal nano-transformation was indicated by the change of colour to light brown. To avoid light and oxygen exposure as far as practicable, the resultant mixture was stored⁴⁰ in the suitably small air-tight amber-coloured bottle without much air space. Product was preserved at 4°C refrigerator before use within three months.

Same synthesis procedure was followed for animal study and cell line study, where 40 mM AgNO_3 and 50 mM of dextrose was used as reducing agent for evaluation of toxicity at ten-folds higher dose in small animal.

Preparation of PVA capped AgNPs as positive control for toxicity study

PVA capped Silver nanoparticles were synthesized by reducing silver nitrate with sodium borohydride and adding polyvinyl alcohol as the capping agent to control the growth of nanocrystals and agglomeration of nanoparticles. Aqueous solutions containing 0.24% (by weight) PVA was heated to 100°C to obtain homogeneity and cooled down to room temperature before use. With 15 mL PVA solution 5 mL of 4 mM AgNO₃ was added to obtain 1 mM solution of silver salt. AgNPs were prepared by rapidly injecting 0.3 mL of 10mM NaBH₄ into 20 mL PVA solution containing 4mM AgNO₃ at room temperature. After 10 min of stirring, the reaction mixture was stored at 4°C before use.

Characterization of AgNPs

The hydrodynamic size of prepared serum-based silver nanoparticles was determined by UV-Vis absorption spectrophotometer (Thermo Scientific Genesys 10S Vis). By lognormal size distribution curves obtained from DLS (Malvern Zen 3600 Zetasizer, USA) measurements, the size ranges of nano mixture with narrow size distributions were indicated. High aggregation stability of such mixture of AgNPs in aqueous dispersion was indicated by suitable negative zeta potential (Malvern Zen 3600 Zetasizer, USA) values. However, the actual size and shape were determined by transmission electron microscope (JEOL JEM 2100 HR with EELS, USA) images. The physical characterization of prepared PVA-capped silver nanoparticles was similarly performed.

Silver nanoparticles formulation

Silver nanoparticles of an average particle size of 17 nm was taken from our synthesized serum capped AgNPs, in a solution form containing about 100 µg silver /mL. The stock solution was first 10fold diluted, then followed two-fold serial dilutions for antimicrobial efficacy study at eight different adjusted two-folds diluted concentrations of 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 µg silver /mL.

Antimicrobial activity and determination of MIC of AgNPs

Extensive antimicrobial studies were conducted for serum-capped AgNPs against test organisms following standard broth dilution method (CLSI M07-A10)⁴¹. The MIC was determined in MH broth using serial two-fold dilutions of Ag-NPs in concentrations ranging from 0.078 ug/mL to 10 ug/mL (silver equivalent) with adjusted bacterial concentration at 0.5 McFarland's standard (0.10 OD at 625 nm) equivalent to about 1×10^8 CFU/mL. The positive control used in this study bacteria inoculated MH broth medium and negative control uninoculated MH broth. The time and temperature of incubation was 24 h and 37°C respectively. The MIC is the lowest concentration of antimicrobial agents that visually inhibits 99% growth of microorganisms. The MIC was noted by the visual turbidity of the tubes both before and after incubation and it was done in five sets to confirm its value for the tested bacteria.

The end-point inhibition levels in two rows containing nano silver solution and equivalent 1/10th. diluted ionic silver solution was noted. Higher antimicrobial actions of AgNPs over equivalent ionic silver were taken as “Bonus Effects” for different bacteria /yeast; those indicated additional microbicidal mechanisms³³.

Statistical analysis

The data thus obtained were analyzed by using descriptive statistics including mean and standard deviation. Non-paired t-Test was done for the analysis of MIC of Ag-NPs against *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans*. Significance of all the statistical tests were predetermined at $P < 0.01$ using Graph Pad Prism version 8.0.2 software.

Effects on conditions for the preservation of nano-antimicrobials

Antimicrobial action of prepared AgNPs was determined at monthly intervals up to six months by observing any change of MIC values for fixed test organism (*Escherichia coli* ATCC-25922) at $\frac{1}{4}$ MIC to 4 MIC range micro-broth dilution study. Stock AgNPs were preserved aerobically at room temperature and refrigeration as well as in hydrogen free oxygen-depleted chamber⁴². Simultaneous five repetitions of the study were done to validate results.

MTT assay on normal liver cell line (WRL 68)

Cell viability was measured by the MTT colorimetric technique with minor adjustment⁴³. In brief, in each well, 200 μ L of MTT [3-(4, 5-dimethylthiazol-2)-2, 5 diphenyl tetrazolium bromide] without phenol red, yellowish in colour solution (5 mg/mL in PBS) was applied to each well containing normal liver cell line in MEM medium with or without varying concentrations of serum-capped AgNPs or PVA-capped AgNPs. Plates were incubated for 6-7 hours in a 5 percent CO₂ incubator for the reduction of MTT by metabolic active cells with formation of purple formazan.

Isopropanol or DMSO was added 100 μ L to each well for the solubilization of MTT crystals. The plates were placed on a shaker for 15 min for complete crystal solubilization and the optical density of each well was then calculated. The quantity of the formazan product was measured by 595 nm using the scanning Multi-well spectrophotometer (Biorad, Model 680, Japan) with DMSO as blank. Absorption OD values proportionately indicated the number of living cells in culture.

Each experiment was performed in duplicate manner. The relative cell viability (percent) of the control well containing the cell culture medium without nanoparticles as a vehicle was determined $A\text{-test} / A\text{-control} \times 100$; where A-test is the absorbance of the test sample and A-control is the absorbance of the control sample.

CFU assay

Human liver cell line (WRL-68) procured from NCCS (Pune, India) was taken for cytotoxicity study. Two mL of MEM medium was added into each well (flat bottom 6 wells tissue culture plate), followed by addition of 10 μ L of confluent cells. After 24 h incubation at 37°C in CO₂ incubator, plates were checked for cell adherence which was continued for another 3 days incubation to observe colony formation. After confirming colony formation, wells were washed with incomplete MEM medium (without FBS). Then, up to 4 mL of fresh complete MEM medium were added with the addition of serum capped 10-fold strong AgNPs, 0 mL (control), 0.37 mL, 0.75 mL and 1.85 respectively. Thus, approximate effective concentrations of silver were achieved to 0, 100, 200 and 500 μ g/mL. at wells in chronological order. Again, after 24 h incubation at 37°C in CO₂ incubator, wells were washed with PBS, then fixed with 500 μ L of methanol for 15 min. Wells were washed twice with PBS, then stained with Hematoxylin for 2 min, followed by washing

in tap water. Cell line imaging was done at 4X inverted microscope. Tests were repeated thrice and CFU % inhibitions in respect of control were calculated.

The margin of a safety study in the animal model

Fifteen male Swiss albino mice of 4-5 months of age with 25-30g body weight were taken, five for every three groups. Tenfold higher AgNPs were prepared for small animal experiment and checked for comparable antimicrobial action at 1/10th dilution. First test group mice were treated with 100 µl of 10-fold-strong serum capped test AgNPs as single intravenous injections, with second untreated group mice as negative control. Third group was treated with 100 µl of AgNPs-PVA containing 1/10th silver equivalent toxic nanoparticles as positive control. By this way, about 107 µg silver equivalent test nanoparticles and 1/10th AgNPs-PVA (10.7 µg silver equivalent) were given per mouse by I/V route which was equivalent to 3570 µg/kg and 357 µg/kg body weight respectively. Their behavioural changes were noted daily, and mice one from each group were sacrificed on 28th day/ on day of death whichever earlier. For histopathological examinations of liver, kidney and brain tissues, each was aseptically dissected. From sections of formal-saline preserved tissues, Eosin-Haematoxylin-stained slides were prepared and examined microscopically to note any haemorrhage and necrotic features difference than that of control.

Biochemical and Hematological Evaluation.

Food was withheld for 24h before necropsy at the conclusion of the 4-week intravenous administration. The mice were anesthetized with Ketamine 22-24 mg/ kg IM route as per CPCSEA guidelines for laboratory animal facility.

Blood was then drawn from ocular puncture, collected in plain vial and analyzed for BUN (blood urea nitrogen); CRE (creatinine); TBIL (total bilirubin); CBIL (conjugated bilirubin); UNBIL (unconjugated bilirubin); ALP (alkaline phosphatase); AST (aspartate amino transaminase); ALT (alanine amino transaminase) using a biochemical blood analyzer.

Ethics Statement

All animal experiments and sample collection were performed at Chittaranjan National Cancer Institute (CNCI). The protocols were approved by the Institutional Animal Ethics Committee (IAEC) at CNCI (Ethical No.- IAEC-1.1/2018/AH-35, 01.09.2017-01.09.2020) and all manuscript confirming the study was carried out in compliance with the ARRIVE guidelines.

REFERENCES:

- 1 Walker B, B. S., Polasky S, Galaz V, Folke C, Engstrom G, Ackerman F, Arrow K, Carpenter S, Chopra K. Environment: Looming global-scale failures and missing institutions. *Science* **325**, 1345-1346 (2009).
- 2 Franci G, F. A., Galdiero S, Palomba L, Rai M, Morelli G and Galdiero M.S Nanoparticles as Potential Antibacterial Agents. . *Molecules* **20**, 8856-8874, doi:10.3390/molecules20058856 (2015).
- 3 Nasrollahi, A., Pourshamsian, K. & Mansourkiaee, P. Antifungal activity of silver nanoparticles on some of fungi. *IJND* **1(3)**, 233-239 (2011).
- 4 Galdiero, S. *et al.* Silver nanoparticles as potential antiviral agents. *Molecules* **16**, 8894-8918 (2011).
- 5 Allahverdiyev, A. M. *et al.* Antileishmanial effect of silver nanoparticles and their enhanced antiparasitic activity under ultraviolet light. *International journal of Nanomedicine* **6**, 2705 (2011).
- 6 Burduşel, A.-C. *et al.* Biomedical applications of silver nanoparticles: An up-to-date overview. *Nanomaterials* **8**, 681 (2018).
- 7 Shrivastava, S. *et al.* Characterization of enhanced antibacterial effects of novel silver nanoparticles. *Nanotechnology* **18**, 225103, doi:10.1088/0957-4484/18/22/225103 (2007).
- 8 Wright, J., Lam, K., Hansen, D. & Burrell, R. Efficacy of topical silver against fungal burn wound pathogens. *American journal of infection control* **27**, 344-350 (1999).
- 9 Garza-Ocañas, L. *et al.* Biodistribution and long-term fate of silver nanoparticles functionalized with bovine serum albumin in rats. *Metallomics* **2**, 204-210 (2010).
- 10 Akter, M. *et al.* A systematic review on silver nanoparticles-induced cytotoxicity: Physicochemical properties and perspectives. *Journal of advanced research* **9**, 1-16 (2018).
- 11 Jorfi, M. & Foster, E. J. Recent advances in nanocellulose for biomedical applications. *Journal of Applied Polymer Science* **132** (2015).
- 12 Kon, K. & Rai, M. Metallic nanoparticles: mechanism of antibacterial action and influencing factors. *J Comp Clin Path Res* **2**, 160-174 (2013).
- 13 Dibrov, P., Dzioba, J., Gosink, K. K. & Häse, C. C. Chemiosmotic mechanism of antimicrobial activity of Ag⁺ in *Vibrio cholerae*. *Antimicrobial agents and chemotherapy* **46**, 2668-2670 (2002).
- 14 Hamouda, T. *et al.* A novel surfactant nanoemulsion with a unique non-irritant topical antimicrobial activity against bacteria, enveloped viruses and fungi. *Microbiological research* **156**, 1-7 (2001).
- 15 Xu, H. *et al.* Role of reactive oxygen species in the antibacterial mechanism of silver nanoparticles on *Escherichia coli* O157: H7. *Biometals* **25**, 45-53 (2012).
- 16 Matsumura, Y., Yoshikata, K., Kunisaki, S.-i. & Tsuchido, T. Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Appl. Environ. Microbiol.* **69**, 4278-4281 (2003).
- 17 Hsin, Y.-H. *et al.* The apoptotic effect of nanosilver is mediated by a ROS-and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells. *Toxicology letters* **179**, 130-139 (2008).
- 18 Netchareonsirisuk, P., Puthong, S., Dubas, S., Palaga, T. & Komolpis, K. Effect of capping agents on the cytotoxicity of silver nanoparticles in human normal and cancer skin cell lines. *Journal of Nanoparticle Research* **18**, 322 (2016).
- 19 Ordzhonikidze, C., Ramaiyya, L., Egorova, E. & Rubanovich, A. Genotoxic effects of silver nanoparticles on mice in vivo. *Acta Naturae (англоязычная версия)* **1** (2009).

- 20 Alshareef, A., Laird, K. & Cross, R. Shape-dependent antibacterial activity of silver nanoparticles on *Escherichia coli* and *Enterococcus faecium* bacterium. *Applied Surface Science* **424**, 310-315 (2017).
- 21 Huang, H. *et al.* An evaluation of blood compatibility of silver nanoparticles. *Scientific reports* **6**, 1-15 (2016).
- 22 Li, S.-Q. *et al.* Nanotoxicity of TiO₂ nanoparticles to erythrocyte in vitro. *Food and chemical toxicology* **46**, 3626-3631 (2008).
- 23 Kwon, T. *et al.* Optimizing hemocompatibility of surfactant-coated silver nanoparticles in human erythrocytes. *Journal of nanoscience and nanotechnology* **12**, 6168-6175 (2012).
- 24 Barkur, S., Lukose, J. & Chidangil, S. Probing Nanoparticle–Cell Interaction Using Micro-Raman Spectroscopy: Silver and Gold Nanoparticle-Induced Stress Effects on Optically Trapped Live Red Blood Cells. *ACS omega* **5**, 1439-1447 (2020).
- 25 Tauran, Y., Brioude, A., Coleman, A. W., Rhimi, M. & Kim, B. Molecular recognition by gold, silver and copper nanoparticles. *World journal of biological chemistry* **4**, 35 (2013).
- 26 Yeh, C.-H., Chen, W.-T., Lin, H.-P., Chang, T.-C. & Lin, Y.-C. Development of an immunoassay based on impedance measurements utilizing an antibody–nanosilver probe, silver enhancement, and electro-microchip. *Sensors and Actuators B: Chemical* **139**, 387-393 (2009).
- 27 Gnanadhas, D. P., Thomas, M. B., Thomas, R., Raichur, A. M. & Chakravorty, D. Interaction of silver nanoparticles with serum proteins affects their antimicrobial activity in vivo. *Antimicrobial agents and chemotherapy* **57**, 4945-4955 (2013).
- 28 Monteiro-Riviere, N. A., Samberg, M. E., Oldenburg, S. J. & Riviere, J. E. Protein binding modulates the cellular uptake of silver nanoparticles into human cells: implications for in vitro to in vivo extrapolations? *Toxicology letters* **220**, 286-293 (2013).
- 29 Sahneh, F. D., Scoglio, C. & Riviere, J. Dynamics of nanoparticle-protein corona complex formation: analytical results from population balance equations. *PLoS one* **8** (2013).
- 30 Van Hong Nguyen, B.-J. L. Protein corona: a new approach for nanomedicine design. *International journal of nanomedicine* **12**, 3137 (2017).
- 31 Saha, K., Moyano, D. F. & Rotello, V. M. Protein coronas suppress the hemolytic activity of hydrophilic and hydrophobic nanoparticles. *Materials horizons* **1**, 102-105 (2014).
- 32 Mirshafiee, V., Mahmoudi, M., Lou, K., Cheng, J. & Kraft, M. L. Protein corona significantly reduces active targeting yield. *Chemical communications* **49**, 2557-2559 (2013).
- 33 Maiti, P. K., Ghosh, A., Parveen, R., Saha, A. & Choudhury, M. G. Preparation of carboxy-methyl cellulose-capped nanosilver particles and their antimicrobial evaluation by an automated device. *Applied Nanoscience* **9**, 105-111 (2019).
- 34 Panáček, A. *et al.* Strong and nonspecific synergistic antibacterial efficiency of antibiotics combined with silver nanoparticles at very low concentrations showing no cytotoxic effect. *Molecules* **21**, 26 (2016).
- 35 Hwang, I.-s., Hwang, J. H., Choi, H., Kim, K.-J. & Lee, D. G. Synergistic effects between silver nanoparticles and antibiotics and the mechanisms involved. *Journal of medical microbiology* **61**, 1719-1726 (2012).
- 36 Ansari, M. A., Khan, H. M., Khan, A., Cameotra, S. & Alzohairy, M. Anti-biofilm efficacy of silver nanoparticles against MRSA and MRSE isolated from wounds in a tertiary care hospital. *Indian journal of medical microbiology* **33**, 101 (2015).

- 37 Maiti, P. K., Parveen, R. & Ghosh, P. EFFECTIVE STEPS TOWARDS THE APPLICATION OF SILVER NANOPARTICLES AS SAFE SYSTEMIC INFECTION CONTROLLING AGENTS. *Paripex-Indian Journal Of Research* **9** (2020).
- 38 Mukherjee, P. *et al.* Green synthesis of highly stabilized nanocrystalline silver particles by a non-pathogenic and agriculturally important fungus *T. asperellum*. *Nanotechnology* **19**, 075103 (2008).
- 39 Song, K. C., Lee, S. M., Park, T. S. & Lee, B. S. Preparation of colloidal silver nanoparticles by chemical reduction method. *Korean Journal of Chemical Engineering* **26**, 153-155 (2009).
- 40 Kittler, S., Greulich, C., Diendorf, J., Koller, M. & Eppele, M. Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. *Chemistry of Materials* **22**, 4548-4554 (2010).
- 41 Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard CLSI document M07-A10, 10th Edn, Wayne, PA: Clinical and Laboratory Standards Institute. CLSI. (2015).
- 42 Maiti, P., Haldar, J., Mukherjee, P. & Dey, R. Anaerobic culture on growth efficient bi-layered culture plate in a modified candle jar using a rapid and slow combustion system. *Indian journal of medical microbiology* **31**, 173 (2013).
- 43 Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods* **65**, 55-63 (1983).

Figure legends

Fig.1. Physical characterization of AgNP-Serum-18 (A) UV–Vis absorption spectra of AgNPs (B) Cluster size obtained from DLS measurements (C) Zeta potential of AgNPs. (D) TEM image

Fig.2. Physical characterization of AgNP-PVA-13 (A) UV–Vis absorption spectra of AgNPs (B) Cluster size obtained from DLS measurements (C) Zeta potential of AgNPs. (D) TEM image

Fig.3: Added anti-microbial action of silver by shifting MIC (log₂ MIC dilution factors or at 2, 4, 8...256, 512 dilutions) following nano conversion [Blue bar ~ 1/10th diluted equivalent Ag⁺ solution]

Fig.4. MTT Assay. (A) Normal liver cell line (WRL-68) with AgNP-Serum-18; (B) Normal liver cell line (WRL-68) with AgNP-PVA-13.

Fig.5. CFU assay. A. Image of a normal human liver cell line (control); B. Cell line with the treatment of AgNPs- Serum-18 at 100 µg/mL silver equivalent concentration; C. Cell line with the treatment of AgNPs at 200 µg/mL silver equivalent concentration; D. Cell line with the treatment of AgNPs at 500 µg/mL silver equivalent concentration; E. Inhibition of colony formation on normal human liver cell line after treatment of AgNPs at various concentrations.

Fig.6. Hematoxylin and eosin (H&E)-stained liver and brain section from mice in different groups. (A) Liver section of mice in the control group showing normal histology and hepatocytes arranged

in the hepatic cords. (B) Liver sections of mice in the group that received 3570 $\mu\text{g/kg}$ AgNP-Serum-18 (I.V), showing: normal histology (C) Liver of mice in the group that received 357 $\mu\text{g/kg}$ AgNP-PVA-13 showing hepatocellular vacuolation and focal area of hemorrhage in the hepatic parenchyma. (D) Brain section of mice in the control group showing normal histology. (E) Brain sections of mice in the group that received 3570 $\mu\text{g/kg}$ AgNP-Serum-18 (I.V), showing: normal histology (F) Brain of mice in the group that received 357 $\mu\text{g/kg}$ AgNP-PVA-13 showing signs of early neuronal injury, focal inflammatory cellular infiltration and vascular congestion. (G) Kidney section of mice in the control group showing normal glomerulus. (H) Kidney section of mice in the Serum capped AgNPs group showing no necrosis (I) Kidney section of mice in the AgNP-PVA-13 group showing necrosis found in kidney tubules.

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Figures

Figure 1

Physical characterization of serum capped silver nanoparticles (A) UV–Vis absorption spectra of AgNPs (B) Cluster size obtained from DLS measurements (C) Zeta potential of AgNPs. (D) TEM image

Figure 2

Physical characterization of PVA capped silver nanoparticles (A) UV-Vis absorption spectra of AgNPs (B) Cluster size obtained from DLS measurements (C) Zeta potential of AgNPs (D) TEM image

Figure 3

Added anti-microbial action of silver by shifting MIC ($\log_2$ MIC dilution factors or at 2, 4, 8,...256, 512 dilutions) following nano conversion [Blue bar ~ 1/10th diluted equivalent Ag⁺ solution]

Figure 4

MTT Assay. (A) Normal liver cell line (WRL-68) with AgNP-Serum-18; (B) Normal liver cell line (WRL-68) with AgNP-PVA-13.

Figure 5

CFU assay. A. Image of a normal human liver cell line (control); B. Cell line with the treatment of AgNPs-Serum-18 at 100 $\mu\text{g}/\text{mL}$ silver equivalent concentration; C. Cell line with the treatment of AgNPs at 200 $\mu\text{g}/\text{mL}$ silver equivalent concentration; D. Cell line with the treatment of AgNPs at 500 $\mu\text{g}/\text{mL}$ silver equivalent concentration; E. Inhibition of colony formation on normal human liver cell line after treatment of AgNPs at various concentrations.

Figure 6

Hematoxylin and eosin (H&E)-stained liver and brain section from mice in different groups. (A) Liver section of mice in the control group showing normal histology and hepatocytes arranged in the hepatic cords. (B) Liver sections of mice in the group that received 3570 $\mu\text{g}/\text{kg}$ AgNP-Serum-18 (I.V), showing: normal histology (C) Liver of mice in the group that received 357 $\mu\text{g}/\text{kg}$ AgNP-PVA-13 showing hepatocellular vacuolation and focal area of hemorrhage in the hepatic parenchyma. (D) Brain section of mice in the control group showing normal histology. (E) Brain sections of mice in the group that received 3570 $\mu\text{g}/\text{kg}$ AgNP-Serum-18 (I.V), showing: normal histology (F) Brain of mice in the group that received 357 $\mu\text{g}/\text{kg}$ AgNP-PVA-13 showing signs of early neuronal injury, focal inflammatory cellular infiltration and vascular congestion. (G) Kidney section of mice in the control group showing normal glomerulus. (H) Kidney section of mice in the Serum capped AgNPs group showing no necrosis (I) Kidney section of mice in the AgNP-PVA-13 group showing necrosis found in kidney tubules.